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Creating Patient-Specific Neural Cells for the In Vitro Study of Brain Disorders

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SUMMARY

As a group, we met to discuss the current challenges for creating meaningful patient-specific in vitro models to study brain disorders. Although the convergence of findings between laboratories and patient cohorts provided us confidence and optimism that hiPSC-based platforms will inform future drug discovery efforts, a number of critical technical challenges remain. This opinion piece outlines our collective views on the current state of hiPSC-based disease modeling and discusses what we see to be the critical objectives that must be addressed collectively as a field.

Just 10 years since the development of human induced pluripotent stem cell (hiPSC) technology (Takahashi et al., 2007), the use of these cells to model brain disorders and obtain disease-relevant information is becoming a tangible reality. Not only are we now able to better detect relevant genetic changes in a patient's cells using high-throughput genome sequencing technology but also we can establish a direct phenotypic correlation between genetic mutations and an aberrant neuronal phenotype or developmental trajectory. The latest improvements in generating relevant neural cell types by either differentiation of hiPSC lines or by direct conversion of somatic cells (e.g., fibroblasts) now allow researchers to make cells from different areas of the central nervous system (CNS) and peripheral nervous system (PNS) and probe effects on the cell

type where disease manifests. This represents a significant improvement of previous experimental tools, including animal models and in vitro cultures of non-relevant cell lines (such as 293T or HeLa cells), which recapitulate only some of the specific traits of human disease (Eglen and Reisine, 2011; Pouton and Haynes, 2005), with the potential to reverse the current trend of huge investments by the pharmaceutical industry yielding few therapeutic compounds entering the market (Mullard, 2015; Scannell et al., 2012).

In April 2015, a group of stem cell researchers, neuroscientists, genomic and computational biologists, clinicians, and industry partners met for 4 days at the Banbury Center at Cold Spring Harbor, New York, to discuss the current challenges for creating meaningful patient-specific in vitro

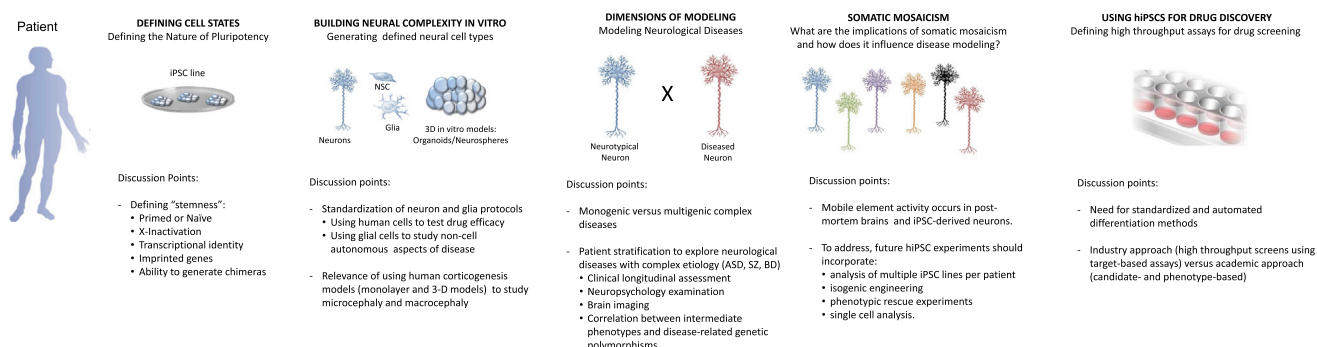


Figure 1. Current Challenges for Creating Meaningful Patient-Specific In Vitro Models to Study Brain Disorders

A critical limitation of the field at present is the inherent difficulty in accurately defining cell states, particularly concerning the temporal and regional identity of pluripotent cells, neurons, and glial cells. A next step for hiPSC-based models of brain disorders will be building neural complexity in vitro, incorporating cell types and 3D organization to achieve network- and circuit-level structures. As the level of cellular complexity increases, new dimensions of modeling will emerge, and modeling neurological diseases that have a more complex etiology will be accessible. An important caveat to hiPSC-based models is the possibility that epigenetic factors and somatic mosaicism may contribute to neurological and neuropsychiatric disease, risk factors that may be difficult to capture in reprogramming or accurately recapitulate in vitro differentiation. A critical next step, in order to enable the use of hiPSCs for drug discovery, will be improving the scalability and reproducibility of in vitro differentiations and functional assays.

models to study brain disorders (Figures 1 and 2). This opinion piece outlines the current state of the field and discusses the main challenges that should drive future research initiatives.

Defining Cell States

The initial discussion at the Banbury meeting addressed the basic properties of stem cells and the increasing appreciation of the heterogeneity of the pluripotent state. The most basic definition of “pluripotency” is the ability of a single cell to differentiate into cells from all three germ layers; however, an improved understanding of the varieties of stem cells and pluripotent states available will broaden the types of cells used as sources for disease modeling and potentially improve production of specific cell types. While we now understand that a variety of artificial stem cell states may be possible during the reprogramming process (Benevento et al., 2014; Clancy et al., 2014; Lee et al., 2014; Tonge et al., 2014), originally, two distinct states of pluripotency were apparent: (1) a “naive” ground state, which was leukemia inhibitory factor (LIF)-dependent, capable of generating both embryonic and extra-embryonic cell lineages, and resembled the properties of mouse embryonic stem cells (mESCs); and (2) a “primed” state, which was FGF2-dependent, reminiscent of “epiblast” identity, and resembled human embryonic stem cells (hESCs) (reviewed by Stadtfeld and Hochedlinger, 2010). In mice, it is well established that inhibition of ERK1/ERK2 and GSK3 β (2i/LIF) is necessary to maintain the naive state (Marks et al., 2012; Ying et al., 2008); withdrawal of 2i/LIF is sufficient to drift naive cells to the

primed state (Brons et al., 2007). Recently, several groups have described culture conditions for maintaining transgene-independent hESCs that share various properties with mESCs (Chan et al., 2013; Gafni et al., 2013; Marinho et al., 2015; Valamehr et al., 2014; Ware et al., 2014). Most compellingly, Hanna and colleagues reported that 2i/LIF, together with EGF, FGF2, JNKi, ROCKi, and p38i, not only converted primed hESCs to the naive state but also conferred competence to form cross-species chimeric mouse embryos (Gafni et al., 2013). While culture of mouse cells in 2i/LIF can convert cells from the primed into the naive ground state, this is not sufficient to convert primed human cells into a naive state. A number of different protocols have been published using a variety of cytokines and inhibitors, with gene expression analyses used to characterize the state of pluripotency. The transcriptome of naive cells generated by some protocols resembled that of mouse naive cells and cleavage human embryos (Takashima et al., 2014; Theunissen et al., 2014), whereas the transcriptome of naive cells produced by other protocols more closely resembled that of primed cells (Brons et al., 2007; Chan et al., 2013; Gafni et al., 2013; Valamehr et al., 2014; Ware et al., 2014). Thus, no consensus on what constitutes the naive human state has been reached, and it is possible that different states of pluripotency exist in human cells. Within this context, a number of presenters considered the importance of carefully defining cell states, in particular the nature of pluripotency.

Rudolf Jaenisch, from the Whitehead Institute for Biomedical Research, reported on iterative chemical screening to evaluate alternative culture conditions



Figure 2. Banbury Meeting Attendees

for naive human pluripotency, ultimately yielding an improved combination of five kinase inhibitors (5i/L/FA) that induces and maintains *OCT4* distal enhancer activity when applied directly to conventional hESCs (Theunissen et al., 2014). Using these optimized conditions, his group demonstrated direct conversion of primed to naive ESCs in the absence of transgenes and isolation of novel hESCs from human blastocysts. They noted, however, that naive hESCs showed upregulated *XIST* and evidence of X inactivation, raising the possibility that X inactivation in naive stem cells in mouse and human may be different. Critically, transplantation of GFP-positive human naive hESCs into mouse blastocysts yielded no GFP-positive E10.5 embryos, either by their original method ($n = 860$ embryos) or by published methods (Gafni et al., 2013) ($n = 436+$ embryos). PCR for human mitochondria is a more sensitive assay, identifying even the presence of 1/10,000 human cells, but this also failed to detect mouse-human chimerism. Although the generation of interspecies chimeras by injection of human ESCs into mouse morulae was proposed as a stringent assay for naive human pluripotency (Gafni et al., 2013), the assay may be too inefficient for use as a routine

functional assay. Instead, Jaenisch suggested that expression profiling is the best method to define naive versus primed ESCs, noting that principal component analysis (PCA) of gene expression from naive hESCs clusters close to mESCs and far from primed hESCs.

Jun Wu, from the Izpisua Belmonte lab at the Salk Institute of Biological Studies, also spoke briefly of recent difficulties in generating viable chimeras following injection of naive human iPSCs tagged with a GFP reporter (hiPSC-GFP). Fortunately, these studies led to media formulations that allowed his group to expand and propagate mouse epiblast stem cell cells (mEpiSCs) from embryonic day 5.75 (E5.75) embryos. When cultured with both FGF2 and WNT inhibition (IWR1), in the absence of serum, these mouse epiblast stem cells showed high cloning efficiency, comparable to that observed in mESCs. Careful characterization revealed a surprising regional specification of these cells (now termed rsEpiSCs); upon transplantation into mouse embryos, although they could contribute to all three germ layers, they could only incorporate into the posterior of the embryo, but not the distal or anterior regions (Wu et al., 2015). Similar culture conditions yielded human



rsPSCs, which also contributed to all three germ layers, exclusively in the posterior region, of chimeric mouse embryos (Wu et al., 2015). This is in sharp contrast to conventional human PSCs, which failed to incorporate in E7.5 mouse epiblast; global genome-wide expression analysis confirmed that these stem cell states have unique molecular signatures.

Ronald McKay, of the Lieber Institute for Brain Studies, further considered molecular regulation of stem cell identity. Sophisticated immunohistochemical analyses revealed unexpected dynamics in the level of pluripotent gene expression, which was high immediately following passaging and declined between splitting and varied between colonies and cultures, relative to their location within the colony (Chen et al., 2014). The transcriptional identity of each stem cell line, however, was stable across datasets and between laboratories, evidence that the dynamic variation between PSCs is defined by our individual human genomes (Adamo et al., 2015). This transcriptional identity not only is conserved in replicate cell lines derived from the same genome but also is stable throughout differentiation; the signature can be detected in post-mortem brain tissue matched to individual stem cell lines. Such signatures may provide a useful means to both classify and assess risk within stratified patient populations without requiring advance knowledge of the target neural cell type(s).

Nissim Benvenisty, from Hebrew University of Jerusalem, discussed epigenetic regulation of stem cells. By generating parthenogenetic hiPSCs from female teratomas that harbor two sets of maternal chromosomes, his group was able to identify novel imprinted genes, including many miRNAs (Stelzer et al., 2011). Rather than observing decreased expression in all paternally expressed genes in parthenogenetic hiPSCs, he reported that about half of the known paternally expressed genes were unexpectedly not downregulated. Two classes of imprinted genes were resolved: the first was downregulated in all parthenogenetic cell types and included classical imprinted genes such as PEG10, whereas the second was not downregulated in some or all examined parthenogenetic cell types and showed overlapping imprinted and non-imprinted isoforms; this resulted from expression from two promoters, only one of which was imprinted (Stelzer et al., 2015). In this context, Benvenisty considered whether parthenogenetic hiPSCs could be used to model epigenetic human disorders such as the neurological Prader-Willi Syndrome (PWS), which results from maternal uniparental disomy of chromosome 15. Characterization of the parthenogenetic PSCs and iPSCs from PWS patients revealed specific maternal expression of the *DLK1-DIO3* locus in chromosome 14. The data suggest that an imprinted gene can work in *trans*, because the loss of expression of *IPW*, an

imprinted long noncoding RNA in the PWS region, is a regulator of *DLK1-DIO3* region. This supports a working model that paternal chromosome 15 mutation in PWS leads to loss of *IPW* and subsequent upregulation of maternal genes (Stelzer et al., 2014).

From these talks arose a discussion of the various tools by which one could define cell states. There was general agreement that genome-wide transcription analysis, both of populations or cells, and particularly at the single-cell level to resolve heterogeneity, was highly informative. Moreover, genetic and epigenetic editing, combined with selective use of cell-line derivation methods, could be tailored to the unique requirements for mechanistic studies of any particular disorder. Finally, as one considers modeling neurological and psychiatric diseases, it is critical that the field as a whole establishes whether or not there is an ideal starting somatic cell type, reprogramming methodology, and/or pluripotency cell state from which to initiate hiPSC-based disease modeling experiments of brain disorders.

Building Complexity to Neuronal Development In Vitro

From here, the focus of discussions turned toward novel methods to generate defined cell types and their application toward a number of highly penetrant neurodevelopmental and neurodegenerative disorders. There was consistent discussion of the critical need to build complexity into hiPSC-based models of neuronal development, first, by more efficiently differentiating and maturing pure populations of neurons, astrocytes, and other neural cell types, and, second, by allowing these populations to self-organize into defined circuits and three-dimensional (3D) systems (organoids) (Eiraku et al., 2008; Kadoshima et al., 2013; Mariani et al., 2012). Earlier work had shown that organoids recapitulate morphogen gradients, cell polarity, layer formation, and other essential features of morphogenesis. Ultimately, there is a need to return to the *in vivo* environment, and a number of researchers discussed early work in transplanting human hiPSC neurons back into either fetal or adult mouse brains (chimeras), in order to track connectivity and systems-level functionality of these cells *in vivo* (Muotri et al., 2005), on the basis of early evidence that hESC-derived human neurons can cross-talk with mouse neurons.

Oliver Brüstle, from the University of Bonn, reported on several stable intermediate neural stem cell populations, which reflect different stages of CNS development and thus facilitate standardized generation of neurons and glia from human pluripotent stem cells (for review, see Karus et al., 2014). The latest addition to this assortment is radial glia-like neural stem cells, which, in contrast to developmentally earlier neural stem cell (NSC) populations, are



endowed with a stable regional identity and enable efficient and more rapid oligodendroglial differentiation (Gorris et al., 2015). Brüstle also gave an update on the StemCell-Factory project, an automated platform for parallelized industrial-scale cell reprogramming and neural differentiation (<http://www.stemcellfactory.de/>). He discussed several applications of PSC-derived NSCs. First, he presented recent comparisons of gamma secretase modulators, finding that amyloid precursor protein (APP) processing in hiPSC neurons is resistant to non-steroidal anti-inflammatory drug (NSAID)-based gamma-secretase modulation (Mertens et al., 2013). This is in contrast to results from transgenic cell lines and mouse models, indicating the need to validate compound efficacy directly in the human cell type affected by disease. Second, Brüstle developed an hiPSC-based model of the polyglutamine disorder Machado-Joseph disease (spinocerebellar ataxia type 3) to illustrate how the earliest steps in protein aggregation can be modeled in patient-derived cells (Koch et al., 2011). Aggregates of ataxin-3 were observed specifically in hiPSC-derived neurons, but not in primary patient fibroblasts, hiPSCs, or hiPSC-derived glial cells. His group's findings indicate that pronounced neuronal intranuclear inclusions are specific to neurons and help to explain the reason for neuron-specific degeneration in this disease. Finally, he also discussed latest developments in studying in vivo integration and connectivity phenotypes of transplanted iPSC-derived neurons with rabies-virus-based monosynaptic tracing and light sheet microscopy of whole-brain preparations.

Allison Ebert, from the Medical College of Wisconsin, described methods for generating astrocyte cultures of improved purity from hiPSCs. In contrasting other recent reports (Emdad et al., 2012; Krencik et al., 2011; Serio et al., 2013), she noted the lengthy duration of existing protocols, which required months to differentiate and expand astrocytes, and she reported on recent attempts to use magnetic activated cell sorting (MACS)-based methods, and even simple cellular passaging, to positively select for astrocyte fate within weeks. Despite some successes, she challenged the field to thoughtfully consider which type of astrocyte each protocol in fact yields and the relevance of these astrocytes to those occurring in vivo. Ebert closed by discussing recent findings from hiPSC astrocyte studies regarding the cell non-autonomous effects underlying reduced synaptic puncti in spinal muscular atrophy (SMA) hiPSC-derived motor neurons (Ebert et al., 2009; Sareen et al., 2012). SMA is a genetic childhood disease characterized by motor neuron loss that is believed to be due to a reduction in the amount of survival motor neuron (SMN) protein in motor neurons. She reported that astrocyte activation could be a non-cell-autonomous contributor to disease, as when SMN is reduced in hiPSC astrocytes and there is increased astrocyte reactivity, and that

co-culture of neurons with SMA astrocytes leads to neuronal phenotype. Together, this work begins to answer why motor neurons are uniquely vulnerable in SMA when SMN is a ubiquitously expressed protein, as it may be that increased astrocyte reactivity ultimately leads to the reduced synaptic puncti observed in SMA hiPSC motor neurons.

Pierre Vanderhaeghen, from the University of Brussels, described efforts to generate defined cortical circuits from hiPSCs (Espuny-Camacho et al., 2013). Their differentiation methods seemed to closely mirror embryonic development, as hiPSCs differentiated first to cortical progenitors, then to pioneer neurons, then to deep layer pyramidal neurons, and finally to upper-layer pyramidal neurons. Although the human timeline was drastically extended, this same pattern was observed in both mouse (1-week) and human (3-month) cells (Nagashima et al., 2014). Using a 3D default differentiation protocol in Matrigel (3DDM differentiation), which yields spheres for analysis within 21 days, Vanderhaeghen's group analyzed lines from subjects with autosomal recessive primary microcephaly (mutations in the *ASPM* gene) (also termed microcephaly primary hereditary [MCPH]). Just as *ASPM* mutations disrupt corticogenesis in the earliest stages, he reported increased neuronal differentiation, although with reduced cortical marker expression, as well as mitotic spindle deviations, in the mutant cells compared to controls. Such phenotypes were not detected in *ASPM* mutant mice, which display only mild microcephaly, suggesting that *ASPM* mechanisms of action may be in part species-specific, underscoring the importance of studying human health in human cells. Moreover, this impairment was not due to the hypothesized defect in proliferation but was more likely the result of perturbed cellular patterning, which could be corrected by applying WNT inhibitor; hence, these models can truly generate novel unexpected mechanistic insights. Finally, Vanderhaeghen reported that PSC-derived cortical cells can be transplanted in neonatal mice, where human neurons develop normally but mature at a considerably slower pace than their mouse counterparts (over 9 months instead of 4 weeks), reminiscent of the neonateness that characterizes neuronal maturation in human cortex.

Madeline Lancaster, from the Institute of Molecular Biotechnology (IMBA) and the MRC Laboratory of Molecular Biology, discussed using cerebral organoids to examine pathogenesis of neurodevelopmental disorders (Lancaster et al., 2013). She noted the many advantages of these self-organizing 3D mixed cultures of human cells, including organized progenitor zones and sequential generation of neuronal layer identities. These organoids comprise radial glia progenitor cells and neurons with good cortical pyramidal morphology. Nonetheless, these



mixed cultures lack axis patterning, show high variability (line to line and batch to batch), and show a loss of neurons with extended differentiation. At their current state of development, organoid assays are likely ideal for studying disorders of neurodevelopment (particularly microencephaly), neurogenesis, and fate specification. Noting that microencephaly is not adequately modeled in rodents, Lancaster, in work performed in the lab of Juergen Knoblich, generated hiPSCs from a microencephaly patient with a null mutation in centrosomal protein *CDK5RAP2* (independent mutations at either allele). She observed a depleted progenitor population and premature neuronal differentiation, demonstrating the precision of this platform in resolving microencephalic phenotypes.

Using a similar strategy, Flora Vaccarino, from Yale University, described applying telencephalic organoids to model early developmental trajectories in autism spectrum disorders (ASD). She noted that human-based studies are critical, owing to fundamental differences in cortical development and timing between humans and mice. Concerns with hiPSCs remain, of course, particularly concerning the potential genetic instability of hiPSCs, which show an accumulation of mutations, tracing back in large part to the original fibroblast population: 30% of skin fibroblasts carry one to two large somatic copy number variants (CNVs), and there is wide variability in the frequency of different mosaic mutations among fibroblast cells (15%–0.3%) (Abyzov et al., 2012). Nonetheless, by applying a neuronal differentiation strategy based upon 3D cortical organoids, Vaccarino demonstrated patient-specific molecular and cellular phenotypes in ASD hiPSC-derived neurons. She reported a methodology for generating cortical organoids that are more homogeneous in structure, composed of repeating units of rosettes, and for which RNA sequencing comparison to the BrainSpan dataset revealed closest similarity to early fetal brain tissue. She cautioned that specific transcriptome differences exist between isogenic intact organoids and dissociated progenitors. Noting that an increase in brain and head size (i.e., macrocephaly) characterizes a subset of ASD patients with poorer outcome, Vaccarino described a study where organoids from patients were systematically compared to those from their fathers in transcriptomics and cellular phenotypes. She reported that ASD hiPSC-derived organoids show a complex cellular phenotype that includes decreased cell cycle length, upregulation of genes directing gamma-amino butyric acid (GABA) neuron fate, increased synaptogenesis and dendrite outgrowth, and changes in synaptic activity. Global gene co-expression network analysis of cortical organoids resolved a number of gene modules that were differentially expressed in ASD individuals, including one potentially driven by *FOXG1*, a master regulatory transcription factor that was greatly upregulated

in ASD. Interestingly, knockdown of *FOXG1* in ASD-derived iPSCs normalized the shift in GABA phenotype in ASD cortical organoids, suggesting a potential causal pathway in the ASD GABAergic imbalance phenotype (Mariani et al., 2015).

Dimensions of Modeling

As the disease modeling field is developing both more reliable in vitro protocols for neural differentiation and more accurate phenotypical functional readouts, researchers are beginning to explore neurological diseases that have more complex etiologies. While highly penetrant monogenic disorders such as Rett and Fragile X syndromes remain among the most tractable areas for iPSC research, the majority of CNS diseases are multigenic, have incomplete penetrance, and are subject to significant environmental influences. One way to circumvent the variability in phenotypes is to stratify the population of patients. Selecting for specific clinical cohorts such as biologically relevant measures, i.e., the brain size phenotype, drug responsiveness, endophenotypes, or specific genetic cohorts containing specific genetic variations with clinical relevance, can provide valuable information toward patient-tailored biomarkers and therapies.

Carol Marchetto, from the Salk Institute of Biological Studies, extended her previous characterization of a monogenic form of ASD (Rett syndrome) (Marchetto et al., 2010) by recruiting a genetically heterogeneous cohort of patients with ASD, characterized by an endophenotype of transient macrocephaly, and comparing them to gender- and age-matched unrelated controls. ASD-derived neural progenitor cells (NPCs) display increased cell proliferation due to a dysregulation of a β -catenin/BRN2 transcriptional cascade, while ASD-derived neurons displayed premature differentiation, reduced synaptogenesis, and altered levels of excitatory and inhibitory neurotransmitters, leading to functional defects in neuronal networks, measured by multielectrode arrays. Interestingly, RNA analysis also revealed increased expression of *FOXG1* in ASD NPCs, in agreement with Flora Vaccarino's data in a completely independent set of experiments, suggesting that there may be common features in macrocephalic ASD and pointing to a potential target of therapeutic intervention.

Kristen Brennand, from the Icahn School of Medicine at Mount Sinai, spoke about the inherent value of modeling predisposition, rather than end-stage disease, in the context of schizophrenia (SZ), noting that gene expression patterns characteristic of SZ hiPSC neurons (Brennand et al., 2011) are conserved in SZ-hiPSC-derived NPCs (Brennand et al., 2015). She presented several phenotypical readouts that would be predictive for SZ predisposition in vitro, such as migration defects (Brennand et al., 2015), WNT signaling defects (Topol et al., 2015), and perturbations in



neuronal connectivity (Brennand et al., 2011) and activity (Yu et al., 2014). By studying the disease phenotype in vitro, she also gained some insight on the disease biology; through the analysis of global expression profiles from SZ-derived NPCs, she reported differential expression of genes and microRNAs related to the migration changes observed in vitro. Additionally, Brennand is working with patient families and a cohort of childhood-onset SZ patients to correlate SZ-related genetic mutations with gene expression levels.

Given the vast clinical heterogeneity of major mental illness, Akira Sawa, from John Hopkins University School of Medicine, advocated careful patient stratification when selecting cohorts for hiPSC-based disease modeling. He argued that traditional *Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)* (American Psychiatric Association, 1994) diagnosis does not provide enough neurobiologically relevant information for patient recruitment for basic research and proposed that other criteria such as clinical longitudinal assessment, neuropsychology examination, brain imaging, and correlation between intermediate phenotypes and disease-related genetic polymorphisms should be considered. By screening olfactory NPCs obtained from a larger clinical cohort of patients with SZ and bipolar disorder (BD) with psychotic features, he identified those patients with reduced phosphorylation (pS713) of disrupted in schizophrenia 1 (DISC1), independent of clozapine treatment, and selected them for further hiPSC-based characterizations. Reduced (pS713) DISC1 phosphorylation was replicated in hiPSC neurons, and levels of p713-DISC1 correlated to neuropsychological and anatomical changes, highlighting the importance of patient stratification in complex neuropsychiatric diseases. He proposed that such clinical phenotype-based stratification of the subjects for hiPSC research could also be applied for unique subsets of SZ and mood disorders, such as psychotic depression and rapid-cycling BD.

Hongjun Song, from Johns Hopkins University School of Medicine, generated hiPSC-derived cortical neurons from four members of a SZ family pedigree defined by a deletion mutation of *DISC1* gene (4-base-pair frameshift deletion on exon 12) (Chiang et al., 2011), observing decreased excitatory postsynaptic current (EPSC) frequencies and synaptic vesicle protein 2 (SV2) puncta density in patients with the mutation, which were rescued by both transcription activator-like effector nucleases (TALEN)-mediated genetic correction (Wen et al., 2014). Subsequent RNA sequencing showed *DISC1*-associated changes in expression of genes involved in neuronal development, synaptic transmission, and those related to mental disorders. Complementary data obtained from genetically modified mice with this same *DISC1* mutation highlighted the continued value of mouse models to study the role of specific mutations

independent of genetic background, as a means of cross-paradigm validation of results obtained with hiPSCs, at the levels of neuronal circuits and behavior, and for in vivo drug testing.

Eric Morrow, from Brown University, showed data from patients with a recently described condition termed Christianson syndrome (CS), a monogenetic X-linked disorder caused by mutations in the endosomal sodium/hydrogen exchanger 6 protein (*NHE6*) (Pescosolido et al., 2014). His laboratory has engineered iPSCs from peripheral blood mononuclear cells from patients with CS and their unaffected male siblings. His studies are investigating a variety of endosomal phenotypes in iPSC-derived neurons as well as cellular phenotypes related to abnormal neuronal differentiation. They are using these cellular assays as platforms to screen candidate treatments. His study emphasized several themes, including pursuing various paths to assemble control cells as well as using statistical methods on experiments on multiple mutations with different subclonal lines. Further, Morrow's studies capitalize on his access to patient clinical assessments, a mouse model, as well as iPSCs. Combining these in vivo studies with the in vitro iPSC studies may prove to be a powerful approach.

Frank Soldner, from the Whitehead Institute for Biomedical Research, having previously generated isogenic hiPSCs at two-point mutations in early-onset Parkinson's disease (PD) (Soldner et al., 2011), now presented studies on the penetrance of non-coding PD risk alleles. Meta-analysis of genome-wide association study (GWAS) data from 13,708 PD cases has identified 26 significant PD-associated loci; however, there is a lack of mechanistic insight in how such sequence variants functionally contribute to complex disease. Soldner proposed that functional disruption of distal enhancer elements leads to the deregulation of gene expression and confers susceptibility to PD. As a proof of principle to study the consequence of these mutations on gene expression, he conducted functional analysis of *cis*-regulatory elements in the *SNCA* locus via genome editing tools in order to precisely disrupt regulatory elements in isogenic pairs. He used quantitative allele-specific assays as readouts and showed that common single nucleotide polymorphisms (SNPs) with small effect size can contribute to PD risk. This work highlights the importance of correlating previously identified disease-related mutations (SNPs and CNVs) with changes in expression profile in vitro in order to identify functional disease-relevant risk variants and determine the mutation's impact.

Rick Livesey, from the Gurdon Institute, provided insights into mechanisms of Alzheimer's disease (AD) pathogenesis using human stem cell models (Shi et al., 2012). The cellular hallmarks of AD are the accumulation of amyloid precursor protein (APP) protein-derived A β peptide fragments and neurofibrillary tangles of the



microtubule-associated protein tau. Livesey described the characterization of hiPSC neurons derived from patients with different familial AD mutations in the APP gene or Presenilin1 (PSEN1), the enzymatic subunit of the γ -secretase complex that processes APP. All of the different mutations increased the release of pathogenic longer forms of A β peptides. However, while increased APP gene dosage and APP mutations all increased total and phosphorylated tau in neurons, PSEN1 mutations did not (Moore et al., 2015). Manipulating γ -secretase activity in human neurons, using available drugs, identified that APP processing is linked to regulating levels of tau protein, hinting that extracellular A β may not be the only process relevant to disease pathogenesis. His work proposes a cell-autonomous link between APP processing and tau.

Lorenz Studer, from Memorial Sloan-Kettering Cancer Center, described work modeling two rare human diseases, familial dysautonomia (FD) and Hirschsprung's disease (colonic aganglionosis). FD is a rare recessive disorder, occurring when a T \rightarrow C point mutation leads to skipping of exon 20 in *iKBKAP/ELP1*. Deriving hiPSCs from patients with both severe (S1 and S2) and mild (M1 and M2) FD, he found that patient-derived hiPSC neurons clearly modeled clinical outcome; relative to unaffected controls, severe FD patients had difficulty generating BRN3A sensory neurons, whereas mild FD patients did not (sensory neurons from both classes of patients die within 28 days). To study Hirschsprung's disease, a fatal if untreated disease in which there is incomplete migration of the enteric nervous system, Studer described a differentiation protocol that successfully generates vagal and enteric neural crest from hESCs that express appropriate cell-type-specific BRN3A/ISL1 markers, produce slow wave activity in vitro, and properly innervate the colon when transplanted into mice (Chambers et al., 2013). hESC-derived *ENRB*^{-/-} and *RET*^{-/-} enteric neural crest cells showed reduced migration in vitro and in vivo. A high-throughput screening (HTS) assay for compounds that rescue these migration deficits identified Pepstatin A. Studer concluded by discussing the technical challenges in studying disorders that require cell types that require significant maturation and aging, some of which are potentially addressable through overexpression of progerin (Miller et al., 2013), as well as methods and assays under-development to address these challenges. For example, he described combining a method to rapidly differentiate cortical neurons with in vivo cell engraftment, in collaboration with Marc Tessier-Lavigne, to yield substantial morphological integration of neurons when imaged by iDISCO, a novel 3D immunohistological processing and imaging technique (Renier et al., 2014); this strategy will allow mapping of the connectivity of human neurons derived from patients with a variety of neurodevelopmental disorders.

Sally Temple, from the Neural Stem Cell Institute, has applied a robust hiPSC differentiation protocol to retinal pigment epithelium (RPE) to understand molecular mechanisms underlying age-related macular degeneration (AMD) (Stanzel et al., 2014), a highly prevalent neurodegenerative disease affecting one in five people older than age 75. A characteristic sign of the early, dry form of AMD is the appearance of large extracellular deposits termed drusen in the macula. Proteomic analysis has demonstrated that drusen share many molecular characteristics with senile plaques in AD. Observing significantly higher expression of AMD and drusen-associated transcripts, particularly A β 42 and A β 40, in AMD iPSC-RPE than in controls, the group took a candidate-based approach and identified several small molecules that reduce AMD-associated transcripts in iPSC-RPE, in some cases irrespective of original AMD disease status. These findings suggest that this in vitro model may be valuable to identify dry AMD therapeutics.

In aggregate, it has become obvious that by more accurately modeling human neurodevelopmental and neurodegenerative diseases in vitro, a number of insights into the cellular and molecular mechanisms underlying the disease state have already arisen. hiPSC-based platforms, combined with genome-scale analyses of sequence variations and transcripts, are increasingly facilitating studies of the temporal dynamics of human disease and allowing researchers to study human-specific elements of disease, asking when critical changes occur in the disease process. From insights into the mechanisms of tau changes in AD, to increased FOXP1 expression in two hiPSC cohorts of ASD, to the critical role of ASPN in microencephaly, cellular models are revealing convergent mechanisms underlying genetically heterogeneous neurological conditions.

Somatic Mosaicism

An emerging issue in the stem cell field is somatic mosaicism, the presence of DNA structural and/or sequence variation from cell to cell in a given individual. This raises interesting questions about not only the role of this form of cellular heterogeneity in health and disease but also the utility of any single patient-derived iPSC line in accurately representing that patient's disease state. Alysso Muotri, from University of California, San Diego, presented data on iPSC-derived neurons from patients with Aicardi-Goutieres syndrome (AGS), a neurodevelopmental disease characterized by intellectual and physical problems with neuroinflammation. Muotri made iPSCs from AGS patients with mutations on *TREX1* gene related to clearance of L1 mobile elements from the cytosol and compared them with isogenic controls. *TREX1*-mutant cells have high levels of single-stranded DNA (ssDNA) derived from mobile retroelements (Alus and L1s) in the cytoplasm and



decreased expression of neuronal markers TLG4, MAP2, TUJ1, and SYN. These features were partially rescued by treatment with reverse transcriptase inhibitors (such as anti-HIV drugs), indicating clinical relevance on this extreme neurological condition. Additionally, *TREX1*-deficient astrocytes also increased ssDNA and triggered an inflammatory response that affected neurons, suggesting a non-cell-autonomous inflammatory effect that may be contributing to neuronal loss in AGS. The *TREX1* mutation highlights the importance of human models, since mouse models for the disease don't present any neurological symptoms.

Mike McConnell, from University of Virginia School of Medicine, presented the use of hiPSC-based neurogenesis to study brain mosaicism (McConnell et al., 2013). His strategy is to perform single cell genomic sequencing. His data from primary brain showed that 45/110 human frontal cortex neurons had megabase-scale CNVs. Similarly, he detected similar levels of mosaic CNVs in hiPSC-derived neurons but very low levels of mosaicism in hiPSC-derived NPCs or human fibroblasts. These data suggest that some aspects of primary brain somatic mosaicism are recapitulated during hiPSC-based neurogenesis. His laboratory is currently defining the levels of genetic mosaicism in neuronal cultures to understand the impact of mosaicism on disease modeling. New data were presented using hiPSC-based neurogenesis to investigate the cause and consequence of brain somatic mosaicism.

It is increasingly clear that somatic mosaicism occurs in both post-mortem and hiPSC neurons. What remains to be determined is the precise extent of this phenomenon in the human brain, its mechanisms, and the precise role that mosaicism contributes to evolution, human behavior and disease, and even the "normal" physiological condition. Moving forward, future hiPSC experiments should be designed with a consideration of the existence of somatic mosaicism, incorporating (1) analysis of multiple iPSC lines per patient, (2) isogenic engineering, and (3) phenotypic rescue experiments.

Using hiPSCs for Drug Discovery

A major goal in the still nascent human stem cell field is to utilize improved cell-based assays in the service of small-molecule therapeutics discovery and virtual early-phase clinical trials. Ajamete Kaykas, from the Novartis Institute for BioMedical Research, discussed the pharmaceutical pipeline to identify phenotypes in human pluripotent stem cell (hPSC)-derived neurons. He demonstrated that in a non-academic setting, it is possible to establish a library of more than 100 transgene-free hiPSCs as well as a clustered regularly interspaced short palindromic repeats (CRISPR) pipeline to create and screen clones for indels, knockout, and point mutation via high-throughput

sequencing methods. In parallel, his group has tested the feasibility of scaling both directed differentiation as well as NGN2-induction protocols into 384-well plate format for high-throughput screening. Overall, both a robust hPSC pipeline for hiPSC-based modeling as well as standardized and automated differentiation methods are being established at Novartis, in collaboration with the Stanley Center, for the characterization and drug screening of SZ patients.

Lee Rubin, from Harvard University, discussed the challenges and successes his laboratory has encountered, in the academic setting, while establishing hiPSC-based high-throughput drug screening for SMA. Given that there are three types of SMA, fatal within the first year of life (type 1), by the teenage years (type 2), and characterized by chronic immobility (type 3), Lee asked whether SMA is in fact one disease or three. To determine why motor neurons from some SMA patients are more sensitive than others, he generated hiPSCs from patients with all three types of SMA, observing that SMA iPSCs have an increased propensity to generate NPCs and a slightly decreased propensity to yield endoderm, consistent with clinical observations that children with SMA have other defects, particularly in endodermal and cardiac tissues. Compared to controls, SMA motor neurons show increased cell death, apoptotic station, reduced neuronal outgrowth, and decreased neuronal firing (SMA3 > SMA2 > SMA1), and regardless of diagnosis, non-motor neurons do not die. Lee conducted three high-throughput screens for compounds to prevent cell death in SMA (ES-derived motor neurons from SMN-deficient mice, SMA patient fibroblasts, and SMA hiPSC-derived motor neurons). Unbiased screens in mouse motor neurons, human motor neurons, and human fibroblasts identified many compounds that increased SMN levels, only some of which overlapped between platforms: while some compounds that block SMN degradation were hits in all three screens, proteasome inhibitors were found in the fibroblast screen but proved toxic to motor neurons (MNs). On the basis of high content imaging data generated through the various screens, his group also observed that at the level of single cells, whether from control or SMA hiPSCs, there is cell-to-cell variation in SMN levels; individual cells with high SMN are the fittest and survive better than neighboring cells with lower SMN, implying that SMN is a general regulator of motor neuron survival, likely owing to reducing activation of the endoplasmic reticulum (ER) stress response. Moreover, inhibitors of the degradation process do not promote survival of SMN neurons below a defined basal level but shift the distribution of SMN, producing more neurons with levels that support survival. On the other hand, compounds that reduce ER stress don't affect SMN levels at all but still promote motor neuron survival. Lee summarized problems



that arise in high-throughput screening of hiPSC-derived cells as those arising due to issues of neuronal variability, immaturity, and non-cell-autonomous disease-relevant interactions.

In discussions among attendees, it became apparent that pharmaceutical and academic scientists approach drug discovery with different perspectives. Traditionally, most academics seek to test the cell type most relevant to disease, pursuing a candidate-based approach to test effects on phenotypes, whereas industry scientists have historically conducted high-throughput screens on entrenched industry-standard screening cell lines using target-based assays. While academics have typically been willing to develop more “risky” assays, the strengths of pharma have historically been in assay selection, scalability, and optimization, as well as drug chemistry and target optimization. Now, research strategies are converging, and both types of researchers are moving toward hiPSC-based screening platforms, drifting toward a hybrid model of testing medium-throughput libraries, screening ~30,000 compound libraries with known targets. New collaborations between academic and pharma researchers promise a future of parallel screening for both targets and phenotypes. Additional hurdles will be encountered if academics are to be the new drivers of drug discovery, including replication (across platforms/reproducibility across sites), documentation (to the rigor of record keeping in industry), and investments to increase automation.

Perspectives and Summary

In line with many of these themes, David Panchision, from the National Institute of Mental Health (NIMH), discussed recent funding initiatives to facilitate cell-based research on mental illness, including those supporting technology development and academic-industry partnerships for developing validated assays. He solicited feedback on NIMH priorities for advancing the field, which involve investigators working together to: (1) implement centralized sharing of patient and reference cell lines with genetic and clinical data, such as through the NIMH Repository and Genomics Resource (<https://www.nimhgenetics.org/>); (2) arrive at common cell-line quality control methods and standards for validating hiPSCs and differentiated cell types; (3) keep improving hiPSC-based technology, including developing easier and quicker targeting methods, optimizing the fidelity of “in vivo” surrogate assays like chimeras and organoids, improving assay miniaturization, and scaling up to increase the number of individuals who can be contrasted by these strategies; (4) focus on robustness and reproducibility, which can include studying genetic variants of large effect from fully characterized patients and maintaining consistency and transparency in protocols/samples across labs; and (5) remain mindful of the critical

value of collaboration and training and supporting the rapid dissemination of best practices (Panchision, 2013).

There was agreement that, although it was important to maximize the rapid sharing and adoption of resources/methods for patient-based disease studies, it was critical that this be balanced with the need for innovation at this early stage in the field. For example, although reprogramming technologies had advanced tremendously, some participants cautioned funding organizations to not restrict iPSC production to a single method or provider, since questions still remained about best practices. Additionally, analysis of specific biological processes or disorders may benefit from tailored cell derivation technologies (e.g., parthenogenesis), stem cell patterning (e.g., naive versus primed hiPSCs), and strategies for genetic manipulation (e.g., CRISPR-Cas9 versus TALEN).

The meeting highlighted the diverse array of cell-based approaches that are being pursued to study human biology and disease, including those (e.g., somatic mosaicism) that illustrated the possibilities and potential limitations of these technologies. Our group was heartened that we are seeing clear disease-related phenotypes in the dish, giving some confidence that discoveries (such as those reflecting early stages in disease processes) would be clinically relevant. Moreover, as such discoveries are being made, unexpected findings are emerging, but the convergence and reproduction among labs improve our group’s optimism that these are robust results and that human cells will be a powerful tool in the therapeutic development armament. Moving forward, a critical application of hiPSC-based studies will be in providing a platform for defining the cellular, molecular, and genetic mechanisms of disease risk, which will be an essential first step toward target discovery. Toward this goal, the case studies discussed demonstrated that different assay systems could yield a surprising convergence of phenotypes, leading to some optimism that the considerable remaining technical challenges to modeling disease are still surmountable.

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All authors contributed to the writing of this report.

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